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JC10 Rec'd PCT/PTO 21 MAR 2002

FORM PTO-1390 (REV 10-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		41377/245565 U S APPLICATION NO (If known, see 37 C F R 1 5) 10/088813
INTERNATIONAL APPLICATION NO PCT/US00/26005	INTERNATIONAL FILING DATE 9/21/00	PRIORITY DATE CLAIMED 9/21/99

TITLE OF INVENTION

METHODS FOR PRODUCING RECOMBINANT PROTEINS

APPLICANT(S) FOR DO/EO/US

Zivko Nikolov

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S C. 371.

3. ☒ This is an express request to promptly begin national examination procedures (35 U S.C 371(f)).

4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31)

5. ☒ A copy of the International Application as filed (35 U S C. 371(c)(2))
   - a ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U S C. 371(c)(3))

9. ☐ An oath or declaration of the inventor(s) (35 U S C 371(c)(4)).

10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))

Items 11. To 16. Below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F R. 1 97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

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U.S. APPLICATION NO (if known, see 37 C F R 1 50) 10/088813	INTERNATIONAL APPLICATION NO PCT/US00/26005	ATTORNEY'S DOCKET NUMBER 41377/245565

17. ☒ The following fees are submitted:		CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,040.00**		
International preliminary examination fee (37 CRF 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$ 890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37CFR 1.445(a)(2)) paid to USPTO	**$ 740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO But all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$ 710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$ 890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		**$**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	47 -20 =	27	X $18.00	$ 486.00	
Independent Claims	3 - 3 =	0	X $84.00	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1376.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by one-half.				$ -688.00	
SUBTOTAL =				$ 688.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 688.00	
Fee for recording the enclosed assignment (37 CFR 1 21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 688.00	
				Amount to be Refunded	$
				Charged	$

a. ☒ A check in the amount of $ 688.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 16-0605 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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Date of Deposit: March 21, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1 10 on the date indicated above and is addressed to BOX PCT, Attn: DO/US (PTO) Commissioner for Patents, Washington, DC 20231.

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WO 01/21270 PCT/US00/26005

METHODS FOR PRODUCING RECOMBINANT PROTEINS

FIELD OF THE INVENTION

The invention relates to the field of biotechnology, particularly to the production of recombinant proteins in plants. The invention further relates to methods for recovering the recombinant proteins from transgenic plants.

BACKGROUND OF THE INVENTION

One of the fundamental achievements of the field of the genetic engineering is the ability to genetically manipulate an organism to produce a protein that the organism was not capable of making prior to human intervention. Typically, the production of such a protein is brought about by facilitating the insertion of a recombinant DNA molecule into an organism. Nucleotide sequences within the recombinant DNA molecule contain the necessary genetic information to direct the host organism to produce the desired recombinant protein. Using such an approach, genetic engineers have modified a variety of eukaryotic and prokaryotic organisms, including bacteria, fungi, animals, and plants, to produce a wide array of recombinant proteins.

Recombinant proteins have had a major impact on agriculture, particularly on crop plants. Recombinant proteins have been used to provide new traits to crop plants which improve their performance in the field. Transgenic corn and cotton plants that have been genetically engineered to produce a bacterially derived insecticidal protein are now widely utilized by farmers. Genetic engineers have also provided the agricultural community with a variety of genetically engineered crop plants that produce proteins which increase a crop plant's tolerance to certain herbicides. Such genetically engineered, herbicide-tolerant soybeans, corn, cotton, and canola are now routinely used in agriculture.

While genetic engineers have achieved resounding successes with the development of such insect-resistant and herbicide-tolerant crop plants, they have not yet reached a similar level of achievement in their attempts to use plants, particularly crop plants, as synthesizers of recombinant proteins for uses such as therapeutic

proteins, industrial enzymes, nutritional supplements, and animal food additives. Several problems have hindered the progress of genetic engineers including insufficient levels of accumulation of recombinant proteins in desired plant tissues and economically inefficient protein extraction methods.

Currently, many recombinant proteins are produced commercially by fermentation utilizing microorganisms. However, fermentative methods are relatively expensive. All inputs necessary for the growth of the microorganisms must be provided including both reduced carbon and nitrogen. Additionally, the microorganisms must be grown under closed, temperature-controlled conditions designed to prevent contamination of the fermentation process with undesired microorganisms.

The production of recombinant proteins in crop plants has the potential to be more cost effective than fermentation, particularly for large-scale recombinant protein production. Because of photosynthesis, plants produce their own reduced carbon, using sunlight, carbon dioxide, and water. Furthermore, crop plant production systems do not involve the expensive facilities that the fermentation of microorganisms require.

However, a major impediment prevents the widespread use of crop plants for recombinant protein production. Difficulties encountered in the extraction of recombinant proteins from plant tissues have made the production of recombinant proteins in plants, for the most part, uneconomical. While difficulties encountered in extracting recombinant proteins from plants may be due to a variety of reasons, often the recombinant protein is produced in cells or parts of cells that make recombinant protein extraction inefficient using current processing technologies. Thus, new methods are needed to take advantage of the potential efficiencies of recombinant protein production in crop plants.

SUMMARY OF THE INVENTION

Methods are provided for producing and recovering recombinant proteins from plant tissues. The methods find use in the biotechnology industry as an efficient means for producing and isolating large quantities of recombinant proteins. Such recombinant proteins can be, for example, therapeutic proteins for humans and other animals, industrial enzymes, and food additives. The methods involve steeping plant

tissues in a solution under conditions favorable for extraction of the recombinant proteins. The methods additionally involve genetically manipulating plants to improve recovery of recombinant proteins from plant tissues by optimizing nucleic acid constructs which comprise a coding sequence of a recombinant protein.

Also provided are plants, plant cells, plant tissues, and seeds thereof that are optimized for the recovery of recombinant proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the effect of steeping solution on aprotinin and corn protein extraction from whole corn kernels after steeping from 0 to 48 hours as described in Example 2. Panel (A) represents the aprotinin and corn protein in steep water. Panel (B) represents the aprotinin and corn protein remaining in the kernels after steeping.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to methods for producing recombinant proteins in plant tissues and recovering the recombinant proteins from the plant tissues. By "recombinant protein" is intended a protein that is produced in an organism as a result of recombinant DNA. The methods find use in the biotechnology industry for producing recombinant proteins such as, for example, industrial enzymes, pesticidal proteins, and proteins used as therapeutic agents, nutritional supplements and food additives for humans and/or animals. The methods of the invention are particularly well suited for use in conjunction with existing grain-processing streams such as, for example, those that make use of wet-milling methodologies. The methods of the invention can be used alone or integrated into existing or newly developed seed-processing systems. Thus, the methods find further use in agriculture by providing producers and processors with a potential new source of income resulting from the production and recovery of recombinant proteins in transgenic crop plants.

The methods of the present invention involve producing and recovering recombinant proteins from plant tissues. By "plant tissue" is intended a whole plant, or any part thereof, including, but not limited to, seeds, organs, and cells. Preferred plant tissues of the invention are plant tissues that produce, or are capable of producing, a recombinant protein therein. More preferred plant tissues are seeds,

fruits, tubers, roots, shoots, leaves, petioles, stems, and flowers, that produce, or are capable of producing, a recombinant protein therein. Most preferred plant tissues are seeds that producem, or are capable of producing, a recombinant protein therein.

Methods are provided for recovering recombinant proteins from plant tissue. The methods comprise producing steep water by steeping plant tissue in a steeping solution. The plant tissue is from a plant that produces recombinant proteins in such a plant tissue. Such a plant is a transgenic plant that possesses a stably integrated nucleic acid construct, particularly a nucleic acid construct, within its genome. The nucleic acid construct comprises a nucleotide sequence encoding the recombinant protein operably linked to a promoter that drives expression in a plant cell. However, any plant tissue containing a recombinant protein can be utilized in the methods of the invention including, but not limited to, plant tissue from a stably transformed plant and plant tissue from a plant that produces recombinant proteins under the direction of recombinant DNA or RNA delivered to a plant by, for example, a virus or a viral vector.

By "steep water" is intended the solution that results from steeping plant tissue in a steeping solution. By "steeping" is intended bringing plant tissue into contact with a solution, herein referred to as a "steeping solution," or the act thereof. Generally, steeping is conducted over a period of time that is determined from such factors as, for example, the plant species, the plant tissue, the steeping solution, the environmental conditions of the steeping, the recombinant protein and the like.

The steeping solution is comprised of water. Additionally, the steeping solution can contain one or more other components including, but not limited to: sulfur dioxide; inorganic acids such as, for example, sulfurous acid, sulfuric acid, phosphoric acid, nitrous acid, nitric acid, hypochlorous acid, hydrochloric acid, carbonic, boric acid, and hydrofluoric acid; organic acids such as, for example, lactic acid, formic acid, succinic acid, malic acid, pyruvic acid, ascorbic acid, malonic acid, tartaric acid, oxalic acid, propionic acid, acetic acid, *n*-butyric acid, isobutyric acid, and citric acid; salts such as, for example, sodium acetate, calcium acetate, potassium acetate, ammonium acetate, magnesium acetate, sodium benzoate, sodium chloride, calcium chloride, potassium chloride, ammonium chloride, magnesium chloride, sodium sulfate, calcium sulfate, potassium sulfate, ammonium sulfate, magnesium sulfate, sodium nitrate, calcium nitrate, potassium nitrate, ammonium nitrate,

magnesium nitrate, sodium nitrite, potassium nitrite, sodium carbonate, calcium carbonate, potassium carbonate, ammonium carbonate, magnesium carbonate, sodium phosphate, calcium phosphate, potassium phosphate, ammonium phosphate, and magnesium phosphate; buffers; chelating agents, antimicrobial agents, preservatives, stabilizers, and the like.

Preferably, such components improve the recovery of the recombinant protein, preserve the desired function or activity of the protein, or both. It is recognized that such a steeping solution can be comprised of process water that originates, for example, in downstream operations commonly used in the corn-refining industry. By "downstream operation" is intended any operation that follows the production of steep water.

Preferred embodiments of the invention make use of whole, unprocessed seeds for producing steep water. However, the methods of the invention also encompass the use of seeds that have been previously processed by one or more methods including, but not limited to, grinding, milling, cracking, defatting, degerminating, fermenting, steaming, heating, cooling, freezing, thawing, pre-soaking in water or other solvents, and the like. Furthermore, the seeds of the invention can be washed or cleaned in some manner prior to steeping to remove or reduce the amount of undesired materials on the surface of the seeds. Such undesired materials include, but are not limited to, soil particles, insects, fungi, spores, and any undesired parts of a plant that are harvested with seeds such as, for example, husks, leaves, cobs, and any part or particles thereof. The seeds can be subjected to any one or more methods for washing or cleaning seeds. Such methods for washing or cleaning seeds can comprise the use of one or more components including, but not limited to, water, a solvent, and a pressured gas or mixture of gases, such as, for example, pressurized carbon dioxide, pressurized nitrogen, and pressurized air. While the washing and cleaning procedures described *supra* are directed toward seeds, those skilled in the art will recognize that other plant tissues of the invention can also be treated in a like manner prior to steeping.

Although the methods of the invention do not depend on a particular volume of steeping solution per unit of plant tissue, those of ordinary skill in the art understand that altering the volume of steeping solution per unit of plant tissue can affect the speed of recovery of the recombinant protein, the total amount of

recombinant protein recovered or both. In addition, the cost of the steeping solution, including any costs of waste-water treatment or disposal, can also be used as a consideration in determining the appropriate volume of steeping solution to use. Thus, the volume of steeping solution depends on the desired outcome. In certain embodiments of the invention, the volume of the steeping solution per bushel of seed is preferably less than about 50 gallons, more preferably less than about 25 gallons, most preferably less than about 10 gallons. Similarly volumes of steeping solution can also be utilized with other plant tissues.

The temperature of steeping can be controlled to improve recovery of the recombinant protein in the steep water. Depending on the recombinant protein, the species of plant, the plant tissue, and the desired outcome, the temperature selected is generally a temperature that allows the maximum recovery of the recombinant protein in the desired form and in the shortest possible time. Typically, such a desired form of a recombinant protein is a form in which the protein is active or capable of performing the intended function such as, for example, an enzymatic activity. Alternatively, a desired form of a recombinant protein can be a non-functional or denatured form. It is recognized that such a denatured form can be renatured at a later time by methods known to those of ordinary skill in the art. Generally, the steeping temperature is less than a temperature which is known to cause coagulation or denaturation of the recombinant protein. Generally, the incubation temperature is greater than the freezing point but less than the boiling point of the steeping solution. Preferably, the incubation temperature is between about 20°C and about 70°C. More preferably, the incubation temperature is between about 35°C and about 65°C. Most preferably, the incubation temperature is between about 40°C and about 60°C.

While preferred methods of the invention employ atmospheric pressure, embodiments of the invention can involve increasing or decreasing the pressure during steeping and during the subsequent separation of the steep water from the steeped plant tissue. Decreasing the pressure during steeping, particularly in the initial phase, can facilitate the uptake of the steeping solution into the plant tissue and thus reduce the length of time of the incubation necessary to achieve the desired recovery of recombinant protein. Increasing the pressure, particularly at the end of steeping when the steep water is withdrawn from the plant tissue, can increase the volume of steep water recovered and thus increase the amount of recombinant protein

recovered. By modifying pressure, increases in the speed of recovery of the recombinant protein, the total amount of recombinant protein recovered or both, can be realized. The methods of the present invention do not depend on any particular method for altering pressure. Any method for altering pressure known to those of ordinary skill in the art can be employed.

The methods of the invention also encompass one or more additional measures known to those of ordinary skill in the art which increase the speed of recovery of the recombinant protein, the total amount of recombinant protein recovered or both. During the steeping of plant tissues, the steeping solution can be, for example, mixed, stirred, agitated, shaken, re-circulated, aerated or de-aerated. The particular additional measures employed, if any, depend on factors such as, for example, the species of plant, the specific plant tissue, the specific recombinant protein and the composition of the steeping solution.

Depending on the desired use of the recombinant protein, further steps can be employed, for example, to concentrate the recombinant protein, to remove impurities from the steeping solution, to separate the desired recombinant protein from undesired proteins and to obtain the recombinant protein in a dry form or in a form in the substantial absence of water. Methods for such steps are known to those of ordinary skill in the art. In addition, one or more components can be added to the steeping solution and/or steep water to preserve and/or stabilize the recombinant protein.

The methods of the invention encompass the use of any protein purification method known in the art. Such methods include, but are not limited to, centrifugation, ultrafiltration, salt precipitation, dialysis, gel-filtration chromatography, ion-exchange chromatography, affinity chromatography, immunoaffinity chromatography, high-performance liquid chromatography (HPLC), reversed-phase high-performance liquid chromatography, ion-exchange high-performance liquid chromatography, size-exclusion high-performance liquid chromatography, high-performance chromatofocusing, hydrophobic interaction chromatography, one-dimensional gel electrophoresis, two-dimensional gel electrophoresis and capillary electrophoresis.

While a desired amount of recombinant protein can be recovered in the steep water, one or more secondary extractions of the steeped plant tissue can be employed in the methods of the invention to increase the recovery of recombinant protein from the plant tissue. By "secondary extraction" is intended any subsequent extraction of a

plant tissue, or any part or parts thereof, that occurs after steeping. In embodiments of the invention involving seeds, preferred seed parts for a secondary extraction include, but are not limited to, an embryo (also referred to as a "germ"), an endosperm, a degerminated seed (i.e. a seed lacking a germ), a seed coat, a tip cap, and a pericarp. Any extraction methods known to those skilled in the art can be employed in such a secondary extraction including, but not limited to, incubating the steeped plant tissue, seed or seed parts in an extraction solution, grinding, and milling. The extraction solution is comprised of water and can additionally contain one or more other components including, but not limited to, the components of a steeping solution described *supra*. Alternatively, the extraction solution is comprised of steep water and can additionally contain one or more other components including, but not limited to, the components of a steeping solution described *supra*. Typically, the recombinant protein is recovered from a secondary extraction in a solution and processed further by any one or more of the additional steps described *supra* for the steep water. The recovered solution can also be combined with the steep water before, during or after any such additional steps are employed. In preferred methods of the invention, the germs, degerminated seeds, or both are subjected to a secondary extraction involving combining the seed parts with an extraction solution comprising steep water.

The methods of the invention make use of any plant tissue that contains a recombinant protein. In preferred embodiments of the invention, the plant tissues produce the recombinant protein under the direction of a nucleic acid construct optimized for recovery of a recombinant protein. Such a nucleic acid construct comprises a nucleotide sequence encoding a recombinant protein operably linked to a promoter that drives expression in a plant cell. Nucleic acid constructs of the invention encompass both DNA constructs and RNA constructs. It is recognized that such DNA and RNA constructs can be either single stranded and double stranded. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases.

By "optimized for recovery" is intended that the nucleotide sequence of the nucleic acid construct has been manipulated by any means known to those of ordinary skill in the art wherein the recovery of a recombinant protein from plant tissue is improved.

By "manipulated" is intended modifying or altering the nucleotide sequence of the nucleic acid construct in any way including, but not limited to, nucleotide substitutions, additions, deletions, inversions, rearrangements and selection of the promoter used to drive expression of the coding sequence of the recombinant protein of the invention.

By "recovery is improved" is intended at least one desired improvement in recovery is achieved. Such a desired improvement in recovery can be, for example, an increase in the level of the recombinant protein in a plant tissue, an increase in the amount of the recombinant protein recovered in the steep water, an increase in the total amount of the recombinant protein recovered in steep water and from a secondary extraction, and an increase in the amount recovered of a desired form of the recombinant protein. Alternatively, a desired improvement can be a reduction in the length or costs of extracting the recombinant protein from plant tissue. Thus, optimizing a nucleic acid construct to improve recovery may or may not lead to an increased amount of a recombinant protein in the plant tissue or an increased amount of a recombinant protein recovered from such a plant tissue.

Most of the corn kernels produced in the United States are processed by the corn-refining industry primarily to extract the starch present in the mature corn kernels. Some of the refined starch is sold as unmodified corn starch or modified into specialty starches prior to sale. However, the majority of the corn starch produced by the corn-refining industry is converted into ethanol, corn syrups, dextrose, and fructose.

The bulk of the corn starch produced in the United States is prepared by the wet-milling process. The first step in the wet-milling process is to steep the corn kernels in an aqueous solution. Steeping the kernels serves two main purposes. First it softens the kernels for subsequent milling, and second, it allows undesired soluble proteins, peptides, minerals and other components to be extracted from the kernels. After steeping, the kernels are separated from the steep water and then wet milled. The steep water is typically concentrated by evaporation to yield a solution referred to as a corn steep liquor. Corn steep liquor typically contains about 3.5 pounds dry solids per bushel of corn kernels with a nitrogen content between 45-48% (Blanchard (1992) *Technology of Corn Wet Milling and Associated Processes*, Elsevier, New York). Protein content in corn steep liquor has been estimated at about one pound per

bushel of steeped corn which amounts to approximately 15-20% (*w/w*) of total corn kernel protein (Blanchard (1992) *Technology of Corn Wet Milling and Associated Processes*, Elsevier, New York). Corn steep liquor is a low-value by-product from the corn wet-milling process and is currently sold as a feed additive or fermentation medium supplement at approximately $50 per ton dry solids.

In a first embodiment of the invention, methods are provided for recovering a recombinant protein from corn kernels in steep water. The methods provide an efficient and economical way to recover recombinant proteins from corn kernels. The cost of recovering recombinant proteins produced in corn kernels by the methods of the present invention is estimated to be approximately $0.50 per kg recombinant protein, assuming that approximately 10% of the dry solids in steep water is recombinant protein. The major advantage of the methods of the invention over existing methods for isolating recombinant proteins from corn kernels is the integration of recombinant protein extraction in the corn wet-milling process. In preferred methods of the invention, kernels steeped by the methods of the invention are suitable for use in the milling or grinding step that occurs after steeping in wet-milling processes. Thus, the costs of retrofitting processing plants and/or adjustments to the corn-refining process are minimized. The methods of the invention also find use with any modified or improved version of the corn wet-milling process that utilizes steeping or any aqueous treatment of corn for the purpose of enhancing corn starch and protein separation. Furthermore, the methods find use in increasing the economic value of corn steep liquor, thus providing the corn-refining industry with a potential new source of profits.

While the composition of the steeping solution, the temperature and duration of steeping, will depend on the recombinant protein and its desired form for recovery, the methods of the present invention involve combining corn kernels with a steeping solution typically containing about 0.1 to about 0.2% sulfur dioxide. Generally, the corn kernels are steeped in such a steeping solution for about 12 to about 48 hours at a temperature of about 50°C. The methods of invention do no depend on steeping kernels for a particular period of time. Typically, kernels are steeped for at least about 1 hour. Preferably, kernels are steeped for at least about 6 hours. More preferably, kernels are steeped for at least about 12 hours. Most preferably, kernels are steeped for about 24 to about 48 hours.

Preferably, the pH of the steeping solution is in the range of about pH 3 to about pH 4, and the volume of the steeping solution per bushel of corn kernels is between about 5 and about 15 gallons. At the end of the incubation, the steep water is withdrawn from the steeped corn kernels. In preferred embodiments of the invention, a desired amount of recombinant protein, preferably in a desired form, is recovered in the steep water, and no further extraction of the steeped kernels is conducted. However, in other embodiments, one or more secondary extractions can be additionally employed to increase the total amount of protein recovered from the kernels.

While sulfur dioxide is routinely employed in existing methods of corn wet milling, the methods of the present invention do not depend on the presence of sulfur dioxide in the steeping solution. In fact, the presence of sulfur dioxide in a steeping solution may be detrimental to recovering certain recombinant proteins in a desired form, particularly those desired forms that depend on one or more disulfide bonds, sulfhydryl groups or both. Such disulfide bonds and sulfhydryl groups may be important for the structure and/or function of a recombinant protein of the invention. The disulfide bonds and sulfhydryl groups of proteins are known to those of ordinary skill in the art and may be involved in functions of a recombinant protein such as, for example, enzyme or catalytic activity, binding activity and channel activity. If desired, the steep solution can contain any one or more of the sulfhydryl reagents typically employed in protein purificaiton methods such as, for example, β-mercaptoethanol, dithiothreitol, and dithioerythritol.

While typical corn wet-milling processes employ a steeping that ranges from 12 to 48 hours, other wet-milling processes such as, for example, those known as the dry-grind process and the intermittent-milling-and-dynamic-steeping process involve an initial steeping of shorter duration and can additional involve steeping at a higher temperature. Typically, the dry-grind and intermittent-milling-and-dynamic-steeping processes involve a steeping of whole kernels for about 12 hours or less at temperatures of about 60°C. The main objective of a such a short initial steeping is to hydrate the embryo or germ. Breaking open the kernel after such a short initial steeping reduces the damage to the germ as compared to dry milling. The hydrated germ can then be recovered by methods typically utilized in the wet-milling process. The degerminated kernel fraction can then be subjected to a second steeping with

additional grinding or milling to facilitate removal of soluble material from the kernel particles. See, Singh and Eckhoff (1996) *Cereal Chem.* 73:716-720 and Lopes-Filho *et al.* (1997) *Cereal Chem. 74*:633-638; herein incorporated by reference.

In a second embodiment of the invention, methods are provided for recovering a recombinant protein from corn kernels comprising producing steep water which comprises steeping kernels for a period of time of less than about 12 hours, preferably less than about 6 hours, more preferably less than about 3 hours, most preferably between about 1 hour and 3 hours. The steeping solution is comprised of water. The steeping solution can additionally contain any one or more of the other components of a steeping solution described *supra*. The recombinant protein of the invention can be recovered in the steep water. Additionally or alternatively, the recovered germ and/or degerminated kernel fraction can be subjected to at least one secondary extraction to recover the recombinant protein. Such a secondary extraction involves combining the germ, degerminated kernel fraction, or both, with an extraction solution, preferably an extraction solution comprising steep water, and incubating the combination.

In preferred methods of the invention, the recombinant protein, particularly a recombinant protein that is expressed in the embryo or endosperm, is recovered from steep water. Such preferred methods can additionally involve a secondary extraction of the recovered germ or degerminated kernel to increase the recovery of the recombinant protein. In such preferred methods, the particle size of the recovered germ or degerminated kernel can be reduced to facilitate recovery of the recombinant protein resulting in, for example, an increased recovery of the recombinant protein from the secondary extraction or a reduction in the duration of the secondary extraction.

In preferred methods of the invention, the corn kernels are from a transgenic corn plant that has a stably integrated nucleic acid construct that has been optimized for recovery of a recombinant protein from kernels. Preferably, such a nucleic acid construct possesses a promoter that directs expression to parts of the kernel that are favored for recovery of the recombinant protein in steep water including, but not limited, to the embryo, endosperm, seed coat, tip cap, and pericarp. The optimized nucleic acid construct can also possess a nucleotide sequence encoding a signal peptide for cell secretion operably linked to the coding sequence of the recombinant protein.

Methods are provided for optimizing a nucleic acid construct for the recovery of a recombinant protein. The methods involve manipulating the nucleotide sequence of a nucleic acid construct to improve recovery of a recombinant protein from plant tissue. The nucleotide sequence of the nucleic acid construct can be manipulated by any means known in the art. The particular manipulations that can be employed depend on factors such as, for example, the particular recombinant protein, the plant species, the plant tissue and the particular process by which the recombinant protein is recovered from the seed. Such manipulations include, but are not limited to, operably linking a promoter that directs expression of the recombinant protein to a desired plant tissue, operably linking a nucleotide sequence that encodes a signal peptide and modifying the nucleotide sequence that encodes the recombinant protein.

Promoters of interest are tissue-preferred, inducible, chemical-regulated, and constitutive promoters. Such tissue-preferred promoters are known in the art and include, but are not limited to, seed-preferred, root-preferred, tuber-preferred, and leaf preferred promoters. Preferred promoters of the invention are those that preferentially direct expression of the recombinant protein to a plant tissue that provides a desired improvement in recovery of the recombinant protein. In methods involving the optimization of a nucleic acid construct for the recovery of a recombinant protein from seeds, preferred promoters are seed-preferred promoters including, but not limited to, promoters that preferentially direct expression to the embryo, the endosperm, the pericarp, the tip cap, the seed coat or combinations thereof.

If desired, the nucleic acid construct can be manipulated in such a manner that the encoded recombinant protein contains the necessary signal for secretion from a plant cell. Typically, a nucleotide sequence encoding a signal peptide is operably linked to the coding sequence of the recombinant protein. Thus, the encoded recombinant protein will contain a signal peptide domain within its polypeptide chain. Such a signal peptide domain directs the secretion of the recombinant protein from a cell and can be removed from or retained in the secreted protein. Within a plant, such a secreted protein is typically found in the cell wall regions or intercellular spaces.

Additionally, the nucleic acid construct can be manipulated to change the nucleotide sequence encoding the recombinant protein. Such changes may or may not alter the amino acid sequence of the recombinant protein. Changes that do not affect the amino acid sequence of the recombinant protein include, for example, codon

optimization. Such codon optimization is known to those skilled in the art and involves changing codons to those preferred for translation by the organism of interest. Preferred codons of an organism are determined by analyzing codon usage frequencies for each amino acid using the coding sequences of cloned genes. Preferred codons for an amino acid are those that are used with the highest frequency in the coding sequences of an organism. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference. Generally, codon optimization involves replacing a non-preferred codon that specifies a particular amino acid with a preferred codon that specifies the same amino acid. Thus, changing such a non-preferred codon to such a preferred codon increases translation in the plant tissue of interest and can increase the amount of recombinant protein in the plant tissue.

The methods of the invention also encompass changes in the nucleotide sequence encoding the recombinant protein. Generally, such changes do not substantially alter the intended function of the protein. Such changes can, however, alter the amino acid sequence of the recombinant protein and include both conservative and non-conservative amino acid substitutions as well as additions and deletions of one or more amino acids. It is recognized that any one or more characteristics or activities of the recombinant protein can be modified including, but not limited to, disulfide bonds, glycosylation sites, myristylation sites, phosphorylation sites, quaternary structure, endoplasmic reticulum retention signals, and catalytic properties such as, for example, substrate specificity, product specificity, K_{cat}, K_m and V_{max}. Furthermore, in the case of recombinant proteins that possess two or more distinct functional domains, one or more of such domains having an undesired function can be removed, or otherwise rendered non-functional, by manipulating the nucleotide sequence encoding the recombinant protein.

Domains can be added to the protein to improve protein recovery. Such a domain can, for example, help stabilize the protein during isolation. Alternatively, such a domain can aid in isolating the protein once it is liberated from plant tissue by protein isolation techniques such as. for example, affinity or immunoaffinity chromatography, and other affinity-based and immunological methods. Such domains include, but are not limited to, a poly-histidine-tag and a domain that interacts with a specific antibody.

To determine if the desired optimization for recovery has been achieved, the nucleic acid construct can, for example, be used to transform a plant. Plant tissue from such a transformed plant or from transformed progeny thereof, is utilized in at least one of the methods of the invention for recovering a recombinant protein from plant tissue. With such an approach, one or more of the various manipulations of the nucleic acid construct described *supra* can be tested singly or in combination for their effect on recovery of a recombinant protein. Those of ordinary skill in the art will recognize that such an approach can be used to select both nucleic acid constructs and plants, optimized for recovery of any recombinant protein.

In a third embodiment of the invention, methods are provided for optimizing a nucleic acid construct for the recovery of a recombinant protein from a grain seed, particularly a corn kernel. The nucleic acid construct is optimized by operably linking the nucleotide sequence encoding the recombinant protein to a promoter that is capable of preferentially directing the expression of the recombinant protein to preferred portions of the corn kernel for improving recovery in steep water. Such a promoter is capable of driving expression in a corn kernel, preferably in the endosperm, embryo, pericarp, tip cap or seed coat of such a corn kernel, more preferably in the embryo, pericarp, tip cap or seed coat, most preferably in the embryo. In preferred methods of the present invention, the nucleic acid construct is further manipulated to operably link a nucleotide sequence encoding a signal peptide for secretion from a plant cell.

A major source of protein in steep water, that is produced by the typical methods utilized in the corn-refining industry for steeping corn kernels before wet milling, is the corn embryo. Additionally, proteins from the embryo are known to appear in the steep water at a relatively faster rate than proteins from other parts of the corn kernel, such as, for example, the endosperm. Thus, preferred methods of the present invention involve a nucleic acid construct comprising a promoter that drives expression preferentially in an embryo.

Methods are provided for optimizing a plant for recovery of a recombinant protein from tissues of the plant. The methods involve stably integrating into the genome of a plant a nucleic acid construct optimized for the recovery of a recombinant protein as described *supra*. The methods find use in providing a plant that is genetically engineered for optimal recovery of a recombinant protein from its

tissues. Such a plant is capable of producing, for example, seeds that contain a recombinant protein, in a desired cellular or subcellular location, in a desired form, or both, for optimal recovery of the recombinant protein from the seed.

In a fourth embodiment of the invention, methods are provided for optimizing a corn plant for recovery of a recombinant protein from kernels comprising stably integrating a nucleic acid construct that is optimized for recovery of a recombinant protein from corn kernels. Preferably, such a nucleic acid construct is optimized for recovery of a recombinant protein from corn kernels essentially as described for the third embodiment *supra*.

Methods for producing a recombinant protein are provided. The methods involve providing a plant with at least one nucleic acid construct comprising a nucleotide sequence encoding a recombinant protein operably linked to a promoter that drives expression in a plant. Such a nucleic acid construct is capable of directing the expression of a recombinant protein within the plant. The methods additionally involve synthesizing the recombinant protein in the plant, harvesting the plant tissue, using the plant tissue to produce steep water by steeping the plant tissue with a steeping solution and recovering the recombinant protein from the steep water.

In a fifth embodiment of the invention, methods are provided for producing a recombinant protein involving stably integrating a nucleic acid construct into the genome of a crop plant, preferably a grain or oilseed plant, more preferably a corn plant. The nucleic acid construct comprises a nucleotide sequence that encodes the recombinant protein operably linked to a promoter that drives expression in a plant cell, particularly a cell in a seed. The methods additionally involve growing the plant, harvesting seeds of the plant and producing steep water by steeping the seeds in a steeping solution. In preferred embodiments of the invention, a desired portion of the total recombinant protein in the seed is recovered in the steep water. Alternatively, if desired, secondary extractions of the steeped seeds can be employed to recover additional recombinant protein. Exemplary embodiments of methods for producing a recombinant protein make use of nucleic acid constructs optimized for recovery of a recombinant protein. Such nucleic acid constructs of the invention are prepared as described *supra*.

Plants transformed with the nucleic acid constructs optimized for recovery of a recombinant protein and seeds thereof, are provided. Transformed plant cells and

tissues are also provided. Such plants and seeds find use in methods for producing, isolating or recovering recombinant proteins in plants and are particularly directed to the optimal recovery of a recombinant proteins from seeds.

The recombinant proteins of the invention comprise any recombinant protein that can be produced in a plant. Recombinant proteins of interest include, but are not limited to, brazzein, avidin, streptavidin, aprotinin, β-glucuronidase, alkaline phosphatase, insulin, bovine somatotropin, human growth hormone, fibrinogen, thrombin, factor IX, factor XIII, serum albumin, plasma proteins, protein C, invertase, superoxide dismutase, catalase, urease, lysozyme, lactase, glucose isomerase, α-amylase, glucoamylase, pullulanase, isoamylase, β-glucanase, xylanase, papain, trypsin, chymotrypsin, pepsin, proteases, protease inhibitors, esterases, peroxidases, hydrolases, phosphatases, kinases, ribonucleases, deoxyribonucleases, antibodies, phytases, lipases, phospholipases, cellulases, hemicellulases, pectinase, peptide hormones, pesticidal proteins, enzymes, and fusion proteins. Of particular interest are soluble, recombinant proteins having commercial value.

Preferably, the recombinant proteins of the invention are selected from industrial enzymes, antibodies, insecticidal proteins, therapeutic proteins, and proteins that are nutritional supplements, nutraceuticals or food additives. More preferably, the recombinant protein is selected from the group consisting of avidin, aprotinin, β-glucuronidase, and brazzein. Most preferably, the recombinant protein is the sweetener protein, brazzein. See, U.S. Patent Nos. 5,326,580; 5,346,998; 5,527,555; and 5, 741,537; herein incorporated by reference.

The recombinant proteins of the invention can be altered in various ways to optimize recovery from plant tissue including, but not limited to, amino acid substitutions, deletions, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the recombinant proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest can be found in the model of

Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference.

In addition, mutagenic and recombinogenic strategies for such as, for example, DNA shuffling can be employed in altering the recombinant proteins of the invention. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The deletions, insertions, and substitutions of the recombinant protein sequences encompassed herein are not expected to produce radical changes in desired characteristics or activities of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays to ensure the continued presence of the desired characteristics or activities.

The use of the term "nucleic acid constructs" herein is not intended to limit the present invention to nucleic acid constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleic acid constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the nucleic acid constructs of the present invention encompass all nucleic acid constructs that can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleic acid constructs of the invention also encompass all forms of nucleic acid constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

The nucleic acid constructs of the invention encompass expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a nucleotide sequence encoding a recombinant protein of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and

mediates transcription of the nucleotide sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The nucleic acid construct can additionally contain at least one additional gene, such as for example, a selectable marker gene.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the coding sequence for the recombinant protein of the invention to be under the transcriptional regulation of the regulatory regions.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a coding sequence for a recombinant protein of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, can be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter can be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced.

In additional to a promoter, the expression cassette can include one or more enhancers. By "enhancer" is intended a cis-acting sequence that increases the utilization of a promoter. Such enhancers can be native to a gene or from a heterologous gene. Further, it is recognized that some promoters can contain one or more native, enhancers or enhancer-like elements.

The termination region can be native with the transcriptional initiation region, can be native with the operably linked DNA sequence of interest, or can be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res. 15*:9627-9639.

Additional sequence modifications are known to enhance gene expression in a plant. These include elimination of sequences encoding spurious polyadenylation

signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes can additionally contain 5'-leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Czech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the nucleic acid construct, the various DNA fragments can be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers can be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

In the methods of the invention, a number of promoters that direct expression of a gene in a plant can be employed. Such promoters can be selected from constitutive, chemical-regulated, inducible, tissue-specific, and seed-preferred promoters. Constitutive promoters include, for example, the core CaMV 35S promoter (Odell *et al.* (1985) *Nature 313*:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-

632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730), and the like. Other constitutive promoters include, for example. U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Inducible promoters can be employed in the methods of the invention such as, for example, chemical-inducible promoters described *supra*, wound-inducible promoters, pathogen-inducible promoters and plant-growth-regulator-inducible promoters. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol. 89*:245-254; Uknes *et al.* (1992) *Plant Cell 4*:645-656; Van Loon (1985) *Plant Mol. Virol. 4*:111-116; Ryan (1990) *Ann. Rev. Phytopath. 28*:425-449; U.S. Patent No. 5,428,148, Rohmeier *et al.* (1993) *Plant Mol. Biol. 22*:783-792; Abe *et al.* (1997) *Plant Cell 9*:1859-1868; Sakai *et al.* (1996) *Plant Cell Physiol. 37*:906-13; Ono *et al.* (1996) *Plant Physiol. 112*:483-91; and Wang and Kutler (1995) *Plant Mol. Biol. 28*:619-34; all of which are herein incorporated by reference.

The preferred promoters of the invention are seed-preferred promoters that are active during seed development. For dicots, seed-preferred promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-preferred promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, γ-zein, waxy, shrunken 1, shrunken 2,

globulin 1, etc. Seed-preferred promoters of particular interest are those promoters that direct gene expression predominantly to specific tissues within the seed such as, for example, the endosperm-preferred promoter of γ-zein, and the embryo-preferred promoter of Glob-1.

The methods of the invention involve providing a plant with a nucleic acid construct comprising a nucleotide sequence encoding a recombinant protein. By "providing" is intended presenting to the plant the nucleic acid construct in such a manner that the construct gains access to the interior of the cell. The methods of the invention further involve the production of the recombinant protein in the plant tissue as a result of the presence of the nucleic acid construct within cells of the plant tissue. The methods of the invention do not depend on a particular method for providing the cells of a plant tissue with such a nucleic acid construct, only that the production of the recombinant protein therein depends on the nucleic acid construct. Methods for providing plants and cells thereof with a nucleic acid construct are known in the art including, but not limited to stable transformation methods, transient transformation methods and viral methods.

By "stable transformation" is intended that the nucleic acid introduced into a plant integrates into the genome of the plant is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleic acid introduced into a plant does not integrate into the genome of the plant.

The nucleic acids of the invention can be provided to the plant by contacting the plant with a virus or viral nucleic acids. Generally, such methods involve incorporating the nucleic acid construct of interest within a viral DNA or RNA molecule. It is recognized that the recombinant protein of the invention can be initially synthesized as part of a viral polyprotein which later can be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Methods for providing plants with nucleic acid constructs and producing the encoded recombinant proteins in the plants, which involve viral DNA or RNA molecules are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

Preferred methods of the invention for providing a plant with a nucleic acid construct involve transforming a plant to stably integrate a nucleic acid construct into the genome of the plant. Transformation protocols as well as protocols for

introducing nucleotide sequences into plants can vary depending on the plant or plant cell targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent stable integration into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Pat No. 5,563,055), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

Generally, the nucleic acid construct will additionally comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Such selectable marker genes and methods of their use in selecting for transformed cells and/or plant tissues are known in the art. Selectable marker genes include, but are not limited to, genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech. 3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:6314-6318; Yao *et al.* (1992) *Cell 71*:63-72; Reznikoff (1992) *Mol. Microbiol. 6*:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell 48*:555-566; Brown *et al.* (1987) *Cell 49*:603-612; Figge *et al.* (1988) *Cell 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:2549-2553; Deuschle *et al.* (1990) *Science 248*:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol. 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res. 19*:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol. 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother. 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry 27*:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother. 36*:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature 334*:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

Transformed plant cells and tissues can be regenerated into plants by standard methods. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting plants producing the desired recombinant protein of the invention. Two or more generations may be grown to ensure that

production of the desired recombinant protein is stably maintained and inherited and then seeds harvested and tested to ensure they possess the desired recombinant protein.

The methods of the invention find use with any plant species capable of producing a recombinant protein. Plants of the invention include, but are not limited to, corn (*Zea mays* or maize), sorghum (*Sorghum bicolor, S. vul*gare), wheat (*Triticum aestivum*), rice (*Oryza sativa*), rye (*Secale cereale*), soybean (*Glycine max*), oats (*Avena sativa*), barley (*Hordeum vulgare*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), canola (*Brassica napus, B. rapa, B. juncea*), oilseed rape (*Brassica spp.*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum, G. barbadense*), flax (*Linum usitatissimum*), peas (*Pisum sativum, Lathyrus spp.*), tobacco (*Nicotiana tabacum*), beans (*Phaseolus spp.*), fava bean (*Vicia faba*), mung bean (*Vigna radiata*), chickpea (Cicer *arientinum*), cowpea (*Vigna sinensis, V. unguiculata*), lentil (*Lens culinaris*), lupines (*Lupinus spp.*), alfalfa (*Medicago sativa*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), peppers (*Capsicum annuum*), sugar beet (*Beta vulgaris*), cassava (*Manihot esculenta*), cocoa (*Theobroma cacao*), carrot (*Daucus carota*), cabbage (*Brassica oleracea var. capitata*), broccoli (*Brassica oleracea var. botrytis*), cauliflower (*Brassica oleracea var. botrytis*), lettuce (*Lactuca sativa*), sweet potato (*Ipomoea batatus*), melons (*Cucumis spp.*), watermelon (*Citrullus lanatus*), squashes (*Curcurbita spp.*), cucumber (*Cucumis. sativus)*, apple (*Malus domestica*), citrus trees (*Citrus spp.*), almond (*Prunus amygdalus*), olive (*Olea europaea*), avocado (*Persea americana*), mango (*Mangifera indica*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), coffee (*Coffea spp.*), guava (*Psidium guajava*), grapes (*Vitus spp.*), millet (*Pennisetum glaucum, Panicum miliaceum, Setaria italica*), *Eleusine coracana*), palms (*Phoenix dactylifera, Elaeis oleifera, E. guineensis*), coconut (*Cocos nucifera*), banana (*Musa* spp.), duckweed (*Lemna spp.*), onion (*Allium cepa*), garlic (*Allium sativum*), and sugarcane (*Saccharum spp.*).

Preferably, the plant species are crop plant species. More preferably, the plant species are selected from the grain and oilseed plants including, but not limited to, corn, sorghum, wheat, millet, rice, rye, soybean, oats, barley, sunflower, safflower, canola, oilseed rape, peanuts, palm, coconut, cotton, and flax. Most preferably, the plant species are corn, wheat, rice, barley, sorghum, canola, cotton, and soybeans.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

Transgenic Corn Plants that Accumulate Aprotinin in Kernels

Aprotinin, the active ingredient in the therapeutic agent, TRASYLOL, is a serine protease inhibitor also referred to as bovine pancreatic trypsin inhibitor. TRASYLOL is indicated for prophylactic use to reduce perioperative blood loss and the need for blood transfusion in patients undergoing cardiopulmonary bypass in the course of coronary artery bypass graft surgery. Currently, commercial preparations of aprotinin are purified from bovine pancreas and lung. However, there is a growing concern that the bovine tissues used to prepare aprotinin may harbor prions that may be pathogenic to humans (Jefferey *et al.* (1995) *Micron 26*:277-298; Smith and Collings (1995) *Essays Biochem. 29*:157-174). Thus, alternative sources of aprotinin are desired.

The production of aprotinin in plants can provide an alternative source of aprotinin for therapeutic preparations such as TRASYLOL. Thus, corn plants were genetically engineered to produce aprotinin in their kernels. An optimized DNA sequence for the aprotinin gene with preferred maize codons was prepared from the known amino acid sequence of the bovine protein. (Anderson and Kingston (1983) *Proc. Natl. Acad. Sci. USA* 80:6838-42). The DNA sequence was optimized for expression in corn by reverse translating the amino acid sequence of the bovine protein using preferred corn codons, and operably linked to a nucleotide sequence encoding a barley α–amylase signal peptide. Such a signal peptide is known to direct the secretion of operably linked proteins from plant cells. Additionally, the maize *ubiquitin* promoter and the potato *pinII* transcriptional terminator were operably linked to the 5' and 3' ends, respectively, of the signal peptide/aprotinin nucleic acid construct. Using this nucleic acid construct, transgenic corn plants were produced that accumulate aprotinin in kernels.

EXAMPLE 2

Recovery of Aprotinin from Kernels of Transgenic Corn Plants by Steeping

Because aprotinin was successfully produced in the kernels of transgenic corn plants, experiments were initiated to develop efficient methods for recovering the recombinant protein from kernels. Preferably, such methods can be integrated into existing corn-refining systems such as those involving, for example, wet-milling processes, dry-grind processes and intermittent-milling-and-dynamic-steeping processes.

Fifty grams of kernels from an aprotinin-expressing, transgenic corn plant described in Example 1 were combined with a steeping solution of either 100 mL water or 100 mL of an aqueous solution of 0.5% lactic acid and 0.2% SO_2 (LA-SO_2). Lactic acid was added to the steeping solution to better simulate the conditions of industrial steeping processes which permit the growth of *Lactobacillus* sp. (Singh and Eckhoff (1996) *Cereal Chem.* 73:716-720; Lopes-Filho *et al.* (1997) *Cereal Chem.* *74*:633-638). The kernels were steeped at 52°C with constant agitation at 200 rpm. At 6, 12, 24, and 48 hours of incubation, duplicate flasks were removed and the steep water was drained from each flask and assayed for total protein by the Bradford method and for aprotinin by an ELISA (enzyme-linked immunosorbent assay). The steeped kernels were air dried and ground, and 2 g of the ground material was extracted with 20 mL of PBS-T (phosphate-buffered saline + Tween 20) to determine the residual levels of total protein (Bradford) and aprotinin (ELISA).

After six hours of steeping, approximately 16 times more aprotinin was recovered in the steep water from the LA-SO_2 treatment than in steep water from the water treatment (Figure 1 and Table 1). By steeping kernels in the LA-SO_2 solution for 24 hours, about 1 μg of aprotinin was recovered in steep water per g of moisture- and oil-free kernel compared to 0.3 μg when water was used. At the end of 24 hours of steeping, the concentration of aprotinin in steep water from the LA-SO_2 and water treatments was 0.1% and 0.05% (*w/w*) of total soluble protein , respectively. As expected more corn protein was extracted during the first 24 hours by steeping with the LA-SO_2 treatment than with the water treatment. However, the 48-hour steeping with water yielded a higher level of corn protein, but not aprotinin, than did the LA-

SO_2 steeping. While higher levels of both corn protein and aprotinin were measured in steep water from the water treatment after 48 hours of steeping than after 24 hours, the opposite was true for the LA-SO_2 samples. After 48 hours of steeping, the levels of both corn protein and aprotinin in steep water from the LA-SO_2 treatment were lower than those measured after 24 hours of steeping.

The residual, extractable levels of both aprotinin and corn protein remaining in the kernels after steeping were determined (Figure 1 and Table 2). The residual levels of aprotinin after steeping with water were 10-20% more than after steeping with LA-SO_2. After six hours of steeping, substantially more corn protein remained in the water-steeped kernels than in the LA-SO_2-steeped kernels. However, when steeping was conducted for more than six hours, the differences in the levels of residual corn protein in between water-steeped kernels and LA-SO_2-steeped kernels were less than 10%.

Table 1

Recovery of Aprotinin by Steeping

Steeping solution	Time (h)	Kernels before extraction			Steep Water*		
		Weight (g)	Oil content (%)	Moisture content (%)	Volume (ml)	Aprotinin (μg/g dry kernel)	Protein (mg/g dry kernel)
Water	6	50	3.4	16	74	0.016	0.09
	12	50	3.4	16	73	0.141	0.43
	24	50	3.4	16	71	0.310	0.65
	48	50	3.4	16	72	0.414	1.05
0.5% lactic acid + 0.2% SO_2	6	50	3.4	16	74	0.262	0.31
	12	50	3.4	16	73	0.547	0.55
	24	50	3.4	16	71	1.047	0.99
	48	50	3.4	16	71	0.965	0.71

*All values are the average of two replicates.

Table 2

Residual Aprotinin and Corn Protein in the Kernels after Steeping

Steeping solution	Time (h)	Kernels after extraction			Residual in kernels*	
		Weight (g)	Oil content (%)	Moisture content (%)	Aprotinin (μg/g dry kernel)	Protein (mg/g dry kernel)
Water	0	2.0	3.4	16	4.483	4.31
	6	2.0	2.7	13	4.406	5.60
	12	2.0	2.0	13	2.557	4.21
	24	2.0	2.0	13	1.616	3.34
	48	2.0	1.8	13	1.545	2.71
0.5% lactic acid +0.2% SO_2	0	2.0	3.4	16	4.483	4.31
	6	2.0	2.2	12	3.542	4.72
	12	2.0	2.2	12	2.055	3.88
	24	2.0	1.7	12	1.247	3.65
	48	2.0	1.6	12	1.070	2.64

*All values are the average of two replicates.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for recovering a recombinant protein from plant tissue comprising steeping said plant tissue with a steeping solution so as to produce steep water; wherein said plant tissue comprises at least one recombinant protein, and said recombinant protein is recovered in said steep water.

2. The method of claim 1, wherein said plant tissue is steeped for at least about 1 hour.

3. The method of claim 1, wherein said plant tissue is steeped for at least about 6 hours.

4. The method of claim 1, wherein said plant tissue is steeped for at least about 12 hours.

5. The method of claim 1, wherein said plant tissue is steeped for at least about 24 hours.

6. The method of claim 1, wherein said plant tissue is selected from the group consisting of seeds, fruits, tubers, roots, shoots, leaves, petioles, stems, and flowers.

7. The method of claim 1, wherein said steeping solution comprises water.

8. The method of claim 7, wherein said steeping solution further comprises at least one component selected from the group consisting of sulfur dioxide, inorganic acids, organic acids, and salts.

9. The method of claim 8, wherein said inorganic acids are selected from the group consisting of sulfurous acid, sulfuric acid, phosphoric acid, nitrous acid,

nitric acid, hypochlorous acid, hydrochloric acid, carbonic acid, boric acid, and hydrofluoric acid.

10. The method of claim 8, wherein said organic acids are selected from the group consisting of lactic acid, formic acid, succinic acid, malic acid, pyruvic acid, ascorbic acid, malonic acid, tartaric acid, oxalic acid, propionic acid, acetic acid, *n*-butyric acid, isobutyric acid, and citric acid.

11. The method of claim 8, wherein said salts are selected from the group consisting of sodium acetate, calcium acetate, potassium acetate, ammonium acetate, magnesium acetate, sodium benzoate, sodium chloride, calcium chloride, potassium chloride, ammonium chloride, magnesium chloride, sodium sulfate, calcium sulfate, potassium sulfate, ammonium sulfate, magnesium sulfate, sodium nitrate, calcium nitrate, potassium nitrate, ammonium nitrate, magnesium nitrate, sodium nitrite, potassium nitrite, sodium carbonate, calcium carbonate, potassium carbonate, ammonium carbonate, magnesium carbonate, sodium phosphate, calcium phosphate, potassium phosphate, ammonium phosphate, and magnesium phosphate.

12. The method of claim 1 further comprising concentrating said steep water.

13. The method of claim 1 further comprising isolating said recombinant protein from said steep water by utilizing at least one technique selected from the group consisting of including centrifugation, ultrafiltration, dialysis, gel-filtration chromatography, ion-exchange chromatography, affinity chromatography, immunoaffinity chromatography, high-performance liquid chromatography, reversed-phase high-performance liquid chromatography, ion-exchange high-performance liquid chromatography, size-exclusion high-performance liquid chromatography, high-performance chromatofocusing, hydrophobic interaction chromatography, one-dimensional gel electrophoresis, two-dimensional gel electrophoresis, and capillary electrophoresis.

14. The method of claim 1 further comprising at least one secondary extraction of said plant tissue, or at least one part thereof.

15. The method of claim 14, wherein said plant tissue is a seed and said part is selected from the group consisting of an embryo, an endosperm, a germ, a degerminated seed, a seed coat, a tip cap, and a pericarp.

16. The method of claim 14, wherein said secondary extraction comprises use of said steep water.

17. The method of claim 1, wherein said plant tissue is from a transgenic plant comprising a stably integrated nucleic acid construct optimized for recovery of said recombinant protein, wherein said nucleic acid construct comprises a nucleotide sequence encoding said recombinant protein operably linked to a promoter that drives expression in a plant cell.

18. The method of claim 17, wherein said nucleotide sequence encodes a protein selected from the group consisting of brazzein, avidin, streptavidin, aprotinin, β-glucuronidase, alkaline phosphatase, insulin, bovine somatotropin, human growth hormone, fibrinogen, thrombin, factor IX, factor XIII, serum albumin, plasma proteins, protein C, invertase, superoxide dismutase, catalase, urease, lysozyme, lactase, glucose isomerase, α-amylase, glucoamylase, pullulanase, isoamylase, β-glucanase, xylanase, papain, trypsin, chymotrypsin, pepsin, proteases, protease inhibitors, esterases, peroxidases, hydrolases, phosphatases, kinases, ribonucleases, deoxyribonucleases, antibodies, phytases, lipases, phospholipases, cellulases, hemicellulases, pectinase, peptide hormones, insecticidal proteins, enzymes, and fusion proteins.

19. The method of claim 17, wherein said promoter is selected from the group consisting of tissue-preferred, seed-preferred, endosperm-preferred, embryo-preferred, inducible, chemical-regulated, and constitutive promoters.

20. The method of claim 1, wherein said recombinant protein is selected from the group consisting of brazzein, avidin, streptavidin, aprotinin, β-glucuronidase, alkaline phosphatase, insulin, bovine somatotropin, human growth hormone, fibrinogen, thrombin, factor IX, factor XIII, serum albumin, plasma proteins, protein C, invertase, superoxide dismutase, catalase, urease, lysozyme, lactase, glucose isomerase, α-amylase, glucoamylase, pullulanase, isoamylase, β-glucanase, xylanase, papain, trypsin, chymotrypsin, pepsin, proteases, protease inhibitors, esterases, peroxidases, hydrolases, phosphatases, kinases, ribonucleases, deoxyribonucleases, antibodies, phytases, lipases, phospholipases, cellulases, hemicellulases, pectinase, peptide hormones, insecticidal proteins, enzymes, and fusion proteins.

21. The method of claim 1, wherein said plant tissue is from a monocot.

22. The method of claim 21, wherein said monocot is selected from the group consisting of corn, wheat, oats, millet, sorghum, rice, barley, rye, palms, coconut, banana, duckweed, onion, garlic, and sugarcane.

23. The method of claim 1 where said plant tissue is from a dicot.

24. The method of claim 23, wherein said dicot is selected from the group consisting of soybean, canola, oilseed rape, cotton, sunflower, safflower, peas, flax, tobacco, beans, fava beans, mung beans, chickpeas, cowpeas, lentils, lupines, alfalfa, potato, tomato, peppers, sugar beet, cassava, cocoa, carrot, cabbage, broccoli, cauliflower, lettuce, sweet potato, melons, watermelon, squashes, cucumber, peanut, apple, citrus, almond, olive, avocado, mango, papaya, cashew, coffee, guava, and grapes.

25. A method for recovering a recombinant protein from a seed comprising steeping said seed, or at least one part thereof, with a steeping solution so as to produce steep water; wherein said seed comprises at least one recombinant protein and said recombinant protein is recovered in said steep water.

26. The method of claim 25, wherein said seed or said part is steeped for at least about 1 hour.

27. The method of claim 25, wherein said seed or said part is steeped for at least about 6 hours.

28. The method of claim 25, wherein said seed or said part is steeped for at least about 12 hours.

29. The method of claim 25, wherein said seed or said part is steeped for at least about 24 hours.

30. The method of claim 25, wherein said seed is a corn kernel.

31. The method of claim 30, wherein, following said steeping, said seed or said part is suitable for wet-milling.

32. The method of claim 25, wherein said steeping solution comprises water.

33. The method of claim 32, wherein said steeping solution further comprises at least one component selected from the group consisting of sulfur dioxide, inorganic acids, organic acids, and salts.

34. The method of claim 25, wherein said seed is from a transgenic plant comprising a stably integrated nucleic acid construct optimized for recovery of said recombinant protein, wherein said nucleic acid construct comprises a nucleotide sequence encoding said recombinant protein operably linked to a promoter that drives expression in a plant cell.

35. The method of claim 25, wherein said recombinant protein is selected from the group consisting of brazzein, avidin, streptavidin, aprotinin, β-glucuronidase, alkaline phosphatase, insulin, bovine somatotropin, human growth hormone,

fibrinogen, thrombin, factor IX, factor XIII, serum albumin, plasma proteins, protein C, invertase, superoxide dismutase, catalase, urease, lysozyme, lactase, glucose isomerase, α-amylase, glucoamylase, pullulanase, isoamylase, β-glucanase, xylanase, papain, trypsin, chymotrypsin, pepsin, proteases, protease inhibitors, esterases, peroxidases, hydrolases, phosphatases, kinases, ribonucleases, deoxyribonucleases, antibodies, phytases, lipases, phospholipases, cellulases, hemicellulases, pectinase, peptide hormones, insecticidal proteins, enzymes, and fusion proteins.

36. A method for producing a recombinant protein comprising:

(a) providing a plant, or at least one cell thereof, with at least one nucleic acid construct comprising a nucleotide sequence encoding said recombinant protein operably linked to a promoter that drives expression in a plant cell;

(b) obtaining plant tissue from said plant, or from a descendant of said plant, wherein said plant tissue comprises said recombinant protein; and

(c) steeping said plant tissue with a steeping solution so as to produce steep water, wherein said recombinant protein is recovered in said steep water.

37. The method of claim 36, wherein said nucleic acid construct is optimized for recovery of said recombinant protein.

38. The method of claim 37, wherein said promoter is selected from the group consisting of tissue-preferred, seed-preferred, endosperm-preferred, embryo-preferred, inducible, chemical-regulated, and constitutive promoters.

39. The method of claim 37, wherein said nucleic acid construct further comprises at least one operably linked element selected from the group consisting of an enhancer, a transcriptional terminator region, a translational terminator region, an intron, exon-intron splice site signals, transposon-like repeats, a translational leader, a polyadenylation signal, and a nucleotide sequence encoding a signal peptide

40. The method of claim 37, wherein said coding sequence is manipulated to alter the amino acid sequence of said recombinant protein.

41. The method of claim 37, wherein said coding sequence is codon optimized.

42. The method of claim 36, wherein said steeping solution comprises water.

43. The method of claim 36, wherein said recombinant protein is selected from the group consisting of brazzein, avidin, streptavidin, aprotinin, β-glucuronidase, alkaline phosphatase, insulin, bovine somatotropin, human growth hormone, fibrinogen, thrombin, factor IX, factor XIII, serum albumin, plasma proteins, protein C, invertase, superoxide dismutase, catalase, urease, lysozyme, lactase, glucose isomerase, α-amylase, glucoamylase, pullulanase, isoamylase, β-glucanase, xylanase, papain, trypsin, chymotrypsin, pepsin, proteases, protease inhibitors, esterases, peroxidases, hydrolases, phosphatases, kinases, ribonucleases, deoxyribonucleases, antibodies, phytases, lipases, phospholipases, cellulases, hemicellulases, pectinase, peptide hormones, insecticidal proteins, enzymes, and fusion proteins.

44. The method of claim 36, wherein said plant tissue is from a monocot.

45. The method of claim 44, wherein said monocot is selected from the group consisting of corn, wheat, oats, millet, sorghum, rice, barley, rye, palms, coconut, banana, duckweed, onion, garlic, and sugarcane.

46. The method of claim 36 where said plant tissue is from a dicot.

47. The method of claim 47, wherein said dicot is selected from the group consisting of soybean, canola, oilseed rape, cotton, sunflower, safflower, peas, flax, tobacco, beans, fava beans, mung beans, chickpeas, cowpeas, lentils, lupines, alfalfa, potato, tomato, peppers, sugar beet, cassava, cocoa, carrot, cabbage, broccoli, cauliflower, lettuce, sweet potato, melons, watermelon, squashes, cucumber, peanut, apple, citrus, almond, olive, avocado, mango, papaya, cashew, coffee, guava, and grapes.

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(54) Title: METHODS FOR PRODUCING RECOMBINANT PROTEINS

(57) Abstract: Methods are provided for producing and recovering recombinant proteins from plant tissue. The methods of the invention find use in the commercial processing of grains, particularly in the wet-milling of corn kernels. The methods involve steeping plant tissue and recovering the recombinant proteins in steep water. The methods additionally involve optimizing nucleic acid constructs and plants for the recovery of recombinant proteins from plant tissue.

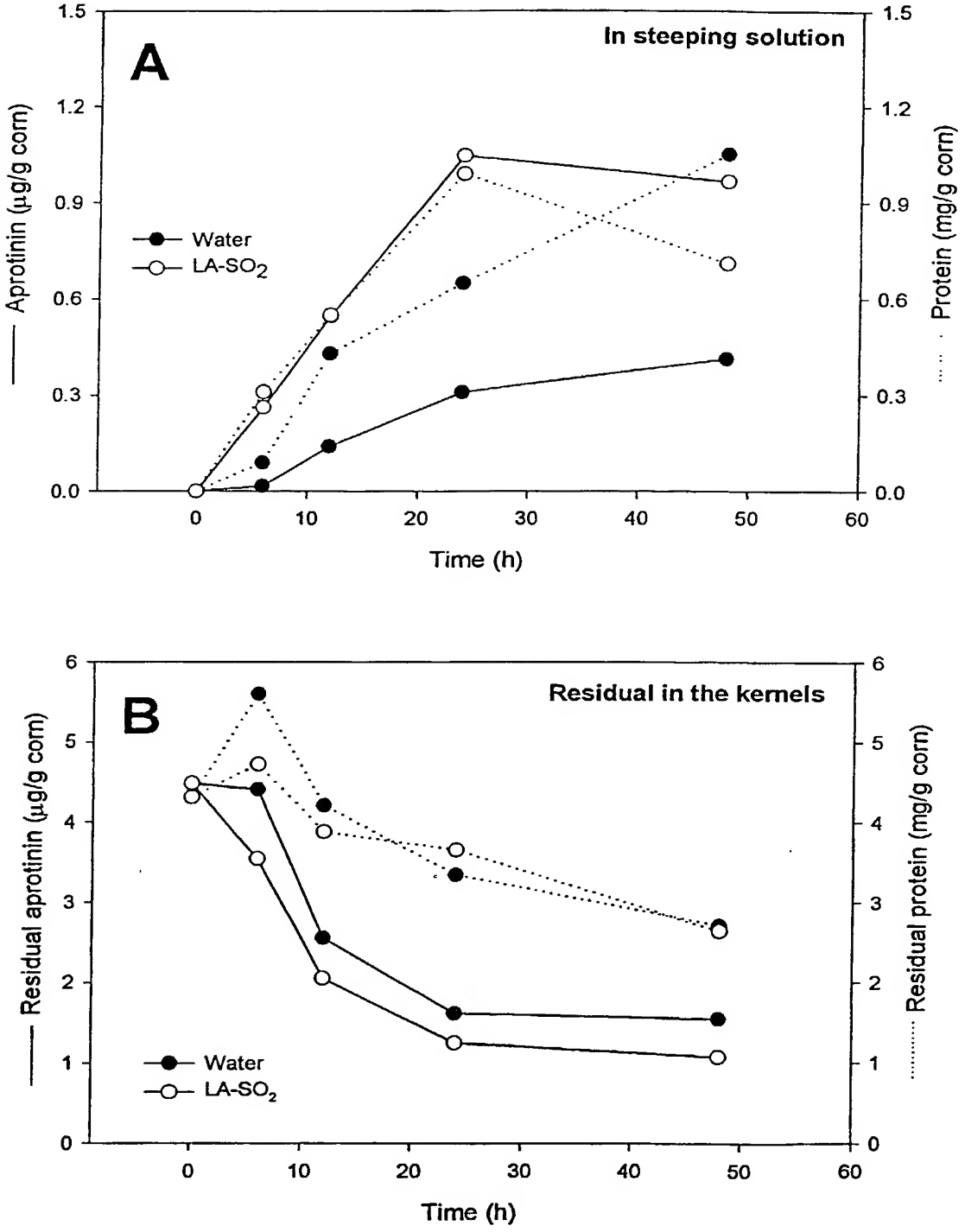

FIGURE 1

Attorney Docket No. 10029

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Zivko Nikolov	
App. No.: 10/088,813	Art Unit:
Filed: March 21, 2002	Examiner:
For: METHODS FOR PRODUCING RECOMBINANT PROTEINS	

POWER OF ATTORNEY

The Commissioner of Patents and Trademarks
Box PCT
U.S. Patent & Trademark Office
Washington, D.C. 20231

Dear Sir:

I hereby appoint, Patricia A. Sweeney, Registration No. 32,733 as my attorney to prosecute this application and to transact all business in the Patent Office connected therewith. All prior powers of attorney are hereby revoked.

It is requested that all correspondence be directed to :

Patricia A. Sweeney
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Respectfully submitted,

By: _____
John Howard, President, ProdiGene, Inc.

==========

CERTIFICATE OF MAILING (37 C.F.R. § 1.8(a))

I hereby certify that this document is being deposited with the United States Postal Service on the date shown below with By Express Mail Post Office to Addressee, Express Mail Number EF285735938 in an envelope addressed to the Commissioner for Patents, Box PCT, U.S. Patent & Trademark Office, Washington, D.C. 20231.

July 11 2002
Date

_____
Patricia A. Sweeney

Attorney Docket No. 10029

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS FOR PRODUCING RECOMBINANT PROTEINS

the specification of which (check one)

☐ is attached hereto
☒ was filed on 21 March, 2002 as Application Serial No. 10/088,813 and was amended on N/A (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filling date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
PCT/US00/26005	PCT	21 September 2000	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56 and, as noted below, where filing a continuation-in-part, such information which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED
60/154,923	21 March 1999	Abandoned

I hereby appoint as my attorney and/agent, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith Patricia A. Sweeney, Registration No. 32,733.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signatures should conform to names as typewritten. ☐ Additional inventors on attached page 3.